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Hazard identification of exhausts from gasoline-ethanol fuel blends using a multi-cellular human lung model

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ABSTRACT

Ethanol can be produced from biomass and as such is renewable, unlike petroleum-based fuel. Almost all gasoline cars can drive with fuel containing 10% ethanol (E10), flex-fuel cars can even use 85% ethanol (E85). Brazil and the USA already include 10–27% ethanol in their standard fuel by law. Most health effect studies on car emissions are however performed with diesel exhausts, and only few data exists for other fuels. In this work we investigated possible toxic effects of exhaust aerosols from ethanol-gasoline blends using a multi-cellular model of the human lung.

A flex-fuel passenger car was driven on a chassis dynamometer and fueled with E10, E85, or pure gasoline (E0). Exhausts obtained from a steady state cycle were directly applied for 6 h at a dilution of 1:10 onto a multi-cellular human lung model mimicking the bronchial compartment composed of human bronchial cells (16HBE14o-), supplemented with human monocyte-derived dendritic cells and monocyte-derived macrophages, cultured at the air-liquid interface. Biological endpoints were assessed after 6 h post incubation and included cytotoxicity, pro-inflammation, oxidative stress, and DNA damage. Filtered air was applied to control cells in parallel to the different exhausts; for comparison an exposure to diesel exhaust was also included in the study.

No differences were measured for the volatile compounds, i.e. CO, NO_x, and T.HC for the different ethanol supplemented exhausts. Average particle number were 6×10^2 #/cm^3 (E0), 1×10^5 #/cm^3 (E10), 3×10^3 #/cm^3 (E85), and 2.8×10^6 #/cm^3 (diesel).

In ethanol-gasoline exposure conditions no cytotoxicity and no morphological changes were observed in the lung cell cultures, in addition no oxidative stress - as analyzed with the glutathione assay - was measured. Gene expression analysis also shows no induction in any of the tested genes, including mRNA levels of genes related to oxidative stress and pro-inflammation, as well as indoleamine 2,3-dioxygenase 1 (*IDO-1*), transcription factor NFE2-related factor 2 (*NFE2L2*), and NAD(P)H dehydrogenase [quinone] 1 (*NQO1*). Finally, no DNA damage was observed with the OxyDNA assay. On the other hand, cell death, oxidative stress, as well as an increase in pro-inflammatory cytokines was observed for cells exposed to diesel exhaust, confirming the results of other studies and the applicability of our exposure system.

In conclusion, the tested exhausts from a flex-fuel gasoline vehicle using different ethanol-gasoline blends did not induce adverse cell responses in this acute exposure. So far ethanol-gasoline blends can promptly be used, though further studies, e.g. chronic and in vivo studies, are needed.

Abbreviations: ALI, Air liquid interface; CO, Carbon monoxide; CVS, Constant volume sampling; E0, pure gasoline; E10, 10% ethanol, 90% gasoline; E85, 85% ethanol, 15% gasoline; GSH, Glutathione; LDH, Lactate dehydrogenase; LSM, Laser scanning microscope; MDDC, Monocyte-derived dendritic cells; MDM, Monocyte-derived macrophages; NO_x, Nitric oxides; PN, Particle number; SSC, Steady state (driving) cycle; T.HC, Total hydrocarbon; WLTC, Worldwide harmonized light vehicle test cycle

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1. Introduction

Petroleum-based fuels will not last forever; renewable sources of energy are therefore needed. Already in the phase of combustion motors invention and development, ethanol was in discussion as a fuel option. But it was not until the 1970s, when ethanol as a fuel supplement started to raise attention, first in Brazil, later also in the USA and Canada. Nowadays, more and more countries recognize ethanol as an interesting alternative or additive to petroleum-based fuels (Agarwal, 2007; Guarieiro and Guarieiro, 2013). Apart from pure gasoline (E0), different ethanol-gasoline blends are used, most prominently 10% ethanol and 90% gasoline (E10), which is the standard gasoline in the USA and planned for the EU by 2020 (EU, 2009), but also 85% ethanol and 15% gasoline (E85) is available, e.g. in Switzerland. While all newer cars can run on E10, special flex-fuel vehicles are needed for E85.

Ethanol acts as a fuel oxygenate, resulting in less particle mass (Chan et al., 2014) and particle number (PN) (Pirjola et al., 2015) in comparison to diesel or gasoline driven vehicles (Guarieiro and Guarieiro, 2013). However, ethanol can also be oxidized to acetaldehyde and further to acetic acid (Lopez-Aparicio and Hak, 2013), and also other aldehydes like formaldehyde and acrolein are expected to be increased in E85 exhaust (Massad et al., 1993). Such emissions are known to be harmful (e.g. Cancer (2006)) and possible effects need therefore to be carefully evaluated (Jacobson, 2007). Aldehydes for example have a very short half-life and are quickly transformed to reactive radicals in the presence of sunlight (Massad et al., 1993). Although research activities have increased during the last years to correlate emissions with engine type and fuel there is still only little data available on the risk assessment in terms of toxicity of ethanol supplemented fuel.

The human lung is especially vulnerable to air pollutants (Loomis et al., 2013) as shown by many epidemiological studies (Brunekreef and Holgate, 2002; Villeneuve et al., 2011; Simkhovich et al., 2008), as well as by human (Ghio et al., 2012; Klepczyńska-Nyström et al., 2012; Larsson et al., 2007; Nyström et al., 2010; Muala et al., 2015), animal (Mauderly et al., 2014; Pardo et al., 2015), and cell culture experiments (Hiraiwa and van Eeden, 2013; Kooter et al., 2013; Oeder et al., 2015; Schwarze et al., 2013; Cheng et al., 2003). Gasoline exhaust was not as extensively studied as diesel exhaust (Reed et al., 2008). The National Environmental Respiratory Center (NERC) published a series of papers on gasoline exhaust (Mauderly et al., 2014; Reed et al., 2008; McDonald et al., 2007). A subchronic gasoline exhaust exposure to rats and mice for 6 months revealed no general health effects like mortality, illness, or injury. However, increased DNA damage in the lung as well as increased cytotoxicity, measured by lactate dehydrogenase (LDH) in the bronchoalveolar lavage has been described. Interestingly a lot of the effects could be attributed to the gaseous phase of the exhaust (Reed et al., 2008). With regard to ethanol exhaust emissions, only studies in the late 80s were found which investigated the health effects of such exhausts (Bernson, 1983; Massad et al., 1985, 1986; Lotfi et al., 1990). Böhm and colleagues exposed rats to gasoline and ethanol exhaust for 5 week and found decreased expiratory flow after gasoline, but not after ethanol exhaust exposure and the most intense pathological lesions in the lungs were observed after gasoline exhaust exposure. Additionally, increased mutagenicity was measured with the micronucleus assay in mice exposed for 2 weeks to gasoline exhaust. These findings, among others, pointed to a chronic toxicity of gasoline exhaust, but not ethanol exhaust (Massad et al., 1986). In addition, the same group also showed that acute toxicity is significantly higher in gasoline than in ethanol exposed animals (Massad et al., 1985).

The attribution of a given subset of emissions to a certain adverse effect is complex but required when different fuels and/or engines are tested. Fast, low-cost, and reliable test systems are therefore needed to assess benefits and risks of these new technologies. For this purpose, the collection of condensable carbonaceous compounds and particle-extractable compounds is possible and is a potential research approach, which has been performed in previous studies (e.g. Che et al., 2010). But, in addition to be labor-intensive, this method does not consider non-condensable gaseous compounds (e.g. nitrogen oxides (NO_x)) and the extraction procedures are likely to affect the samples. A more accurate method consists of collecting the complete exhaust, including the particulate, condensed, and gaseous fraction. To address these issues we have developed a method which allows exposing human lung epithelial cells cultured at the air-liquid interface (ALI) directly to the complete engine exhaust. This system has already been used in the past for risk assessment of scooter (Muller et al., 2010), diesel (Steiner et al., 2012, 2013a. 2013b, 2014) and gasoline (Bisig et al., 2015) exhaust.

The aim of this study was to investigate the exhaust components produced from a passenger car with ethanol supplemented fuels ranging from E0, E10, and E85 and to correlate the emissions with possible effects in a multi-cellular human lung model. The lung cells were exposed to the exhaust at the ALI for 6 h and after a 6 h post-incubation biological endpoints such as cytotoxicity, pro-inflammation, oxidative stress, and mutagenicity were assessed. The effects were compared to filtered air as well as to cells exposed to diesel vehicle exhaust without a particle filter.

2. Materials and methods

2.1. Materials

All materials were purchased from Sigma-Aldrich unless otherwise stated.

2.2. Cell cultures

The multi-cellular human lung model composed of three cell types mimicking the bronchial compartment was used as previously described (Steiner et al., 2013a). Briefly, 16HBE14o- human bronchial epithelial cells (Cozens et al., 1994; Forbes et al., 2003) were seeded (2.4×10^5 cells/cm^2) on fibronectin-coated 6-well inserts (3 μm pores, BD Falcon) and cultured for 5 days. Human whole blood monocytes were isolated from buffy-coats provided by the blood donation service SRK Bern and purified using CD14 Microbeads (Milteny Biotech, Bergisch Gladbach, Germany) as described by Steiner et al. (2013b). Monocytes were differentiated to macrophages (MDM) and dendritic cells (MDDC) for 5–6 days and then added on top (1.2×10^4 MDM cells/cm^2) and on the bottom (2.0×10^5 MDDC cells/cm^2) of the insert, respectively. Differentiation agents were GM-CSF (Granulocyte macrophage colony-stimulating factor, Milteny Biotech) and IL-4 (Biotechne, R & D systems, Abington, United Kingdom) to obtain MDDC and M-CSF (Macrophage Colony-Stimulating Factor, Milteny Biotech) for MDM, all at a concentration of 10 ng/ml. One day after composition, the multi-cellular model was transferred to the ALI for another day before exposure to either filtered air or exhaust.

2.3. Test vehicle and exposure system

A modern gasoline flex-fuel passenger car (2012, Euro5a, gasoline direct injection, mileage during exposures was 10′000–15′000 km) was driven on a chassis dynamometer for 6 h. With the exception of the fuel, no changes to the car were made; inclusively standard lubricant oil (Castrol Magnatec 5 W-30) and the original three-way catalyst were used in all conditions. E85-fuel was purchased at a gas station and E10 was obtained by splash blending commercial E0 (RON 95) and E85. The fuels were stored at room temperature in 60 l barrels for less than 2 months. Unlike the mixture of diesel and non-esterified plant oils, ethanol and gasoline mix very well and no separation is expected.

A steady state cycle (SSC), consisting of five states (each 20 min with 95 km/h, 61 km/h, 45 km/h, 26 km/h, and idling) was driven;

these velocities are derived from the Worldwide harmonized light vehicle test cycle (WLTC) (UNECE, 2016), representing the mean velocity of each of the four parts. In a 6 h exposure, the SSC was repeated 3 times, the fourth cycle was started until 6 h were completed. The SSC was chosen not only because of its simplicity, but also because an internal study comparing the new European driving cycle (NEDC, (European_Parliament, 1997)) against the SSC only found marginal differences in cell responses (unpublished data). Velocity-time diagrams are shown in Fig. S1.

The test-order was as follows: E10, E85, and in the end E0. It is important to note that E0 was driven for other experiments before this test series. The car was not adjusted for the new fuel before the experiment started and not used for anything else between the exposures.

As a positive control, a diesel car (1998, Euro2) was driven the WLTC for 6 h. Standard market diesel, the original lubricant oil, and the oxidative catalyst were used. No additional after-treatment systems were installed (e.g. no particle filter).

The exposure chamber set-up was used as described earlier (Muller et al., 2010). Briefly the exhaust was diluted ten times and pumped through the cell culture chamber with a flow of 2 L/min. In a parallel chamber, filtered air was applied with the same flow. Cell environment was kept identical in both chambers, approximately 85% relative humidity (rH), 5% CO_2, and 37 °C. Though keeping identical rH in filtered air and E85 exposure was difficult to achieve, due to the humidity of E85-exhaust.

2.4. Exhaust characterization

The exposure experiments and hence the online exhaust characterization were performed at the exhaust gas control station of the Bern University of Applied Sciences in Nidau, Switzerland, an institution officially accredited for exhaust gas control by the Swiss government. Exhaust sample characterization was performed in parallel to the exposure experiments, yielding detailed information on the exhaust the cells were exposed to. The PN was measured in the 10-fold diluted exhaust using a condensation particle counter (TSI 3790). The lower particle size detection characteristics of TSI 3790 are 50 ± 12% at 23 nm (D50 efficiency) and > 90% at 41 nm (D90 efficiency), with maximal detectable particles at > 3 µm. Particle number and size distribution were measured for each velocity with a scanning mobility particle sizer (SMPS, differential mobility analyzer, TSI 3081, range of 10–415 nm). Furthermore, the concentrations of carbon monoxide (CO), carbon dioxide (CO_2), total gaseous hydrocarbons (T.HC), and NO_x were measured using a Horiba MEXA-9400H exhaust gas measuring system.

From each driving cycle (SSC or WLTC), averages were calculated per day of exposure, data is presented as median (interleaved low-high) of the obtained averages (n=4 for SSC, n=6 for WLTC). The flame ionization detector for T.HC measurements was calibrated using propane; therefore T.HC-values of E85 were corrected by a factor of 0.758.

2.5. Cell sample analyses

After the 6 h post incubation time, cells and supernatants from the triple cell co-culture were collected, and processed as described for the specific assays. Supernatants were stored in the fridge or freezer for later analysis. All cells within the multi-cellular model were collected together; differentiation between the three different cells used in the different assays was therefore not possible.

2.5.1. Quantification of lactate dehydrogenase (LDH) release

LDH is a ubiquitous protein and can easily be measured in the supernatant when membrane damage occurred with the LDH detection kit (Cytotoxicity Detection Kit (LDH), Roche Applied Science). The kit was used according to the manufacturer's protocol; the positive assay control was done with 0.25% Triton X-100.

2.5.2. Laser scanning microscopy (LSM)

Microscopy images were taken to evaluate cell morphology after the different exposures. Samples were fixed in 3% paraformaldehyde for 5–10 min and stored at 4 °C in phosphate buffered saline (PBS) for later staining. Before staining, samples were washed with PBS, permeabilized with 0.25% Triton X-100 for 15 min, washed again, then labelled with Phalloidin Rhodamine (F-Actin stain) and 4′,6-diamidino-2-phenylindole (DAPI, nuclei stain) for one hour. After a last washing step samples were mounted on objective slides in Glycergel®. Image acquisition was performed on a Zeiss LSM 710. Image restoration was done with the IMARIS software (Bitplane 7.4, Zürich, Switzerland).

2.5.3. Quantification of total glutathione (GSH)

GSH, an anti-oxidative stress tri-peptide neutralizes reactive oxygen species. The assay was performed according to the manufacturer's protocol. Briefly, samples were lysed and deproteinized directly after the post incubation and stored in at −20 °C for later visualization of GSH with the glutathione assay kit (Cayman Chemical, provided by Adipogen AG, Liestal, Switzerland). The GSH concentrations were normalized to total protein as measured with the bicinchoninic acid assay according to manufacturer's protocol (BCA protein assay kit, Pierce). The positive assay control to induce oxidative stress was tert-butyl hydroperoxide (tBHP, 500 µL, 5 mM in PBS), which was added to the cells for 12 h.

2.5.4. Gene expression analysis

Reverse real time polymerase chain reaction (RT-PCR) allows the quantification of genes on a transcriptional level. RNA isolation was performed with the RNeasy plus kit (Qiagen). cDNA was produced with the Omniscript RT system (Qiagen), Oligo dT (Microsynth), and RNasin Inhibitor (Promega) as recently described by Bisig et al. (2015). RT-PCR was done with SYBR-green (Applied Biosystems) on a 7500 Fast Real-Time PCR (Applied Biosystems).

Data was calculated using the $^{\Delta\Delta}$Ct method, glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) being the standard gene and filtered air exposure the control sample. Heme oxygenase 1 (*HMOX1*), superoxide dismutase 2 (*SOD2*), and glutathione reductase (*GSR*) were genes used to assess oxidative stress. For pro-inflammatory responses, genes for tumor necrosis factor α (*TNFα*) and interleukin-8 (*IL-8*) were measured. Additionally three genes were assessed in this study, indoleamine 2,3-dioxygenase 1 (*IDO-1*), transcription factor NFE2-related factor 2 (*NFE2L2*), and NAD(P)H dehydrogenase [quinone] 1 (*NQO1*). The primer sequences for all assessed genes can be found in SI Table S2.

2.5.5. OxyDNA adducts

An indirect marker for genotoxicity is the amount of 8-oxoguanine adducts formed on DNA (DNA damage). Using the OxyDNA Assay Kit (Merck Millipore, Nottingham, UK), 8-oxoguanine adducts were fluorescently labelled and measured with flow cytometry (FACS). A detailed protocol as well as the results can be found in the SI.

2.6. Data processing and statistical analysis

2.6.1. Number of repetitions

For each exhaust, four times 6 h were driven (n=4), but eight different cultures with cells from different passage numbers and monocyte isolations were exposed in total, to achieve higher statistical power (more information in SI Table S1). Unless otherwise stated, statistical analysis was performed with n=8. For the diesel control exposure, cells were exposed on six different days (n=6, unless otherwise stated).

2.6.2. Data normalization

Cultures from the same day were paired and exposed to either exhaust or filtered air. For the assessed bioassays, except gene expression analysis, data obtained from the exhaust exposed cells was divided by the paired cells exposed to the filtered air. This normalization was performed to counter different background levels of different blood donors and non-exhaust related effects. Positive assay controls (chemicals) were paired with an untreated control (left in incubator).

2.6.3. Data presentation

In order to give the reader an unbiased view of the results, all normalized data points are presented as single points with the mean. Since the results are normalized (fold changes), the *Y*-axis is plotted in Log2-scale. In the text, fold changes relative to the control (filtered air or untreated control) are presented as mean ± stdv.

Statistical analysis was performed on Prism 6.01 and *p*-values < 0.05 were considered as statistically significant. Two way ANOVA and Sidak's multiple comparison tests were used to test for differences between the filtered air exposure and its correspondent exhaust exposure. One way ANOVA and Tukey's multiple comparison test showed differences between the different exhaust types (which were normalized to filtered air).

3. Results

3.1. Exhaust characterization

Different standard exhaust components (i.e. CO, T.HC, NO_x, and PN) were measured and are shown in Fig. 1A-D. The volatile fractions CO, T.HC, and NO_x did not differ between the different exhausts of ethanol-gasoline blends. Notably very low concentrations of NO_x were measured (Fig. 1C), 0.11 ± 0.06 ppm (E0), 0.08 ± 0.08 ppm (E10), and 0.06 ± 0.04 ppm (E85). CO concentrations were 6.3 ± 0.4 ppm (E0), 6.1 ± 0.6 ppm (E10), and 6.5 ± 0.3 ppm (E85), in the same range were the T.HC concentrations, 5.2 ± 0.3 ppm (E0), 5.1 ± 0.7 ppm (E10), and 3.9 ± 0.3 ppm (E85). In the filtered air we measured 0.01–0.06 ppm NOx, 1.4–2.0 ppm CO, and 2.8–4.9 ppm THC.

The produced PN varied significantly for the different fuels, E0 and E85 exhaust emitted on average 600 ± 260 #/cm^3 and 2500 ± 4700 #/cm^3, respectively. E10 exhaust showed up to 200x higher PN concentration ($1.27 \pm 0.06 \times 10^5$ #/cm^3) in comparison to E0 and E85. SMPS data is shown in Fig. S3, and confirms the low PN in E0 and E85. Particle mass was not measured, but is estimated to be 18.9 µg/m^3 in E10, assuming spherical shape and a particle density of 1.2 g/cm^3 (Maricq and Xu, 2004). Diesel exhaust analysis revealed significantly higher NO_x concentration (52.6 ± 0.7 ppm) and PN ($2.8 \pm 1.3 \times 10^6$ #/cm^3), while the levels of CO (2.8 ± 0.7 ppm) and T.HC (5.4 ± 0.2 ppm) remained comparable to the flex-fuel vehicle.

3.2. Biological endpoints

Microscopy images showed no changes in morphology and the epithelial cells revealed a homogenous and confluent distribution (Fig. 2A XY plane). A monolayer of epithelial cells on the apical side (Fig. 2A XZ plane) and MDDC on the basolateral side (not shown) of the inserts were observed. Release of LDH by the cells was increased for diesel exhaust exposure (1.4 ± 0.7 fold change) and significantly increased for the positive assay control Triton-X (7.0 ± 1.8 fold change), but no increase in the other exhaust types was observed (normalized to filtered air). E85 exhaust even significantly reduced LDH level by 0.7 ± 0.2 compared to filtered air (Fig. 2B).

The GSH level, a direct marker for oxidative stress, decreased significantly after diesel exhaust (0.2 ± 0.2) and the positive assay

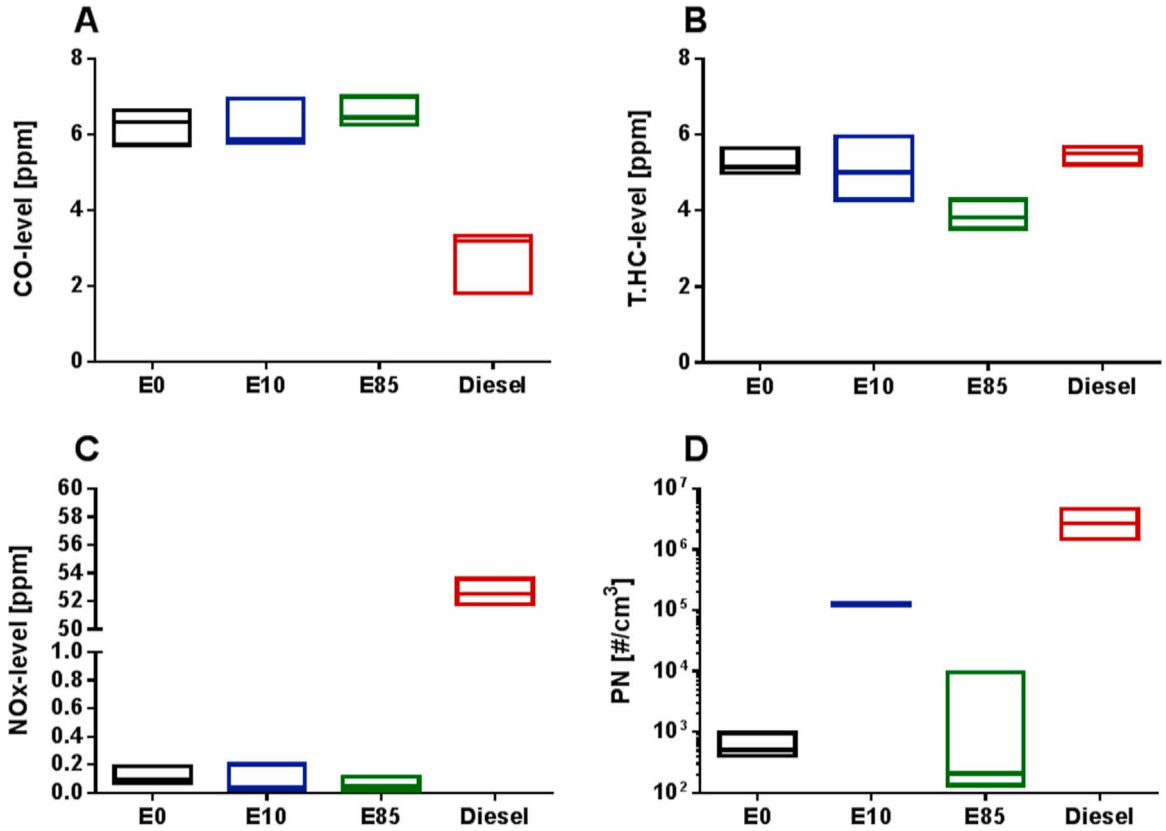

Fig. 1. Exhaust analysis. (A-C) Volatile fraction measured in the CVS tunnel. CO= carbon monoxide (A); T.HC= total hydrocarbons (B); NO_x= nitric oxides (C). (D) Particulate fraction, measured in the ten-fold diluted exhaust. Average PN of all velocities are 600, 127′000, 2500, and 2′770′000 #/ cm^3 for E0, E10, E85, and diesel, respectively. Data are shown interleaved low-high with line at median. N=4 for the flex-fuel vehicle (SSC), n=6 for diesel (WLTC).

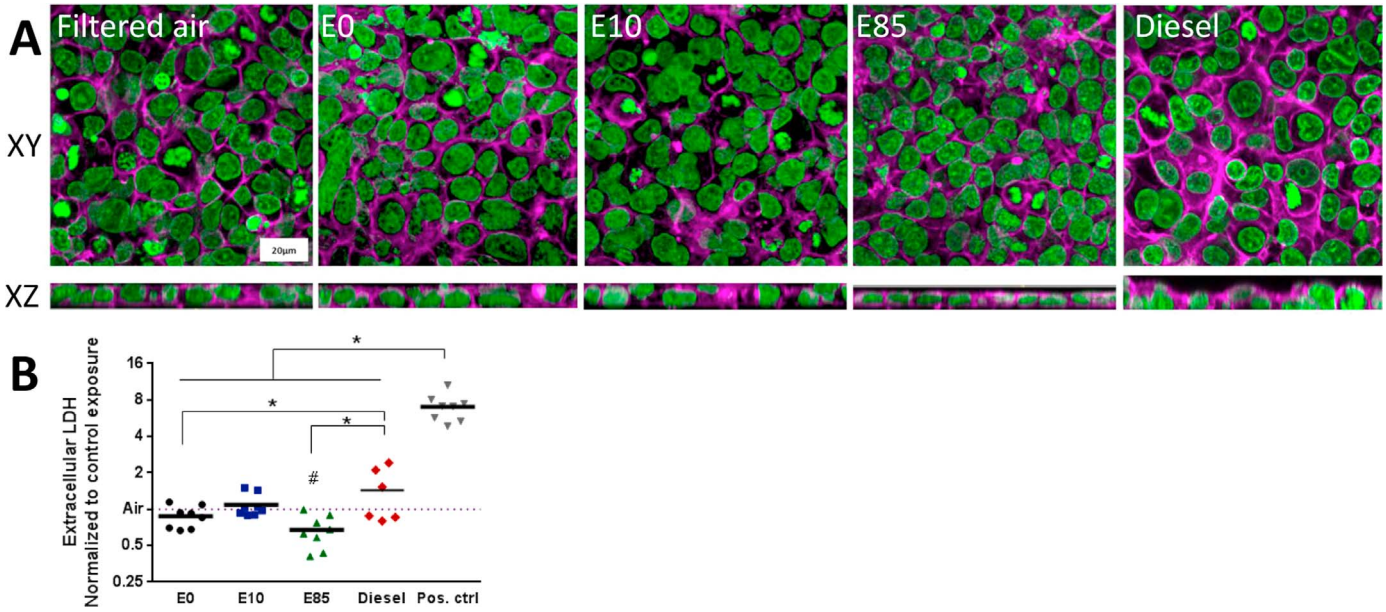

Fig. 2. Cell viability and cell morphology after exhaust exposures. (A) Representative microscopy images from XY and XZ projections. Cells were stained with phalloidin rhodamine (F-Actin cytoskeleton, magenta) and DAPI (cell nuclei, green). No morphological changes are observed. (B) Extracellular LDH levels, significantly higher LDH was measured after diesel exhaust compared to E0 and E85, indicating some cytotoxicity in diesel exhaust. Data are shown as single values and mean (line);* depict differences between exposures, # between filtered air and its correlating exposure, $p < 0.05$ was considered statistically significant.

control tBHP (0.6 ± 0.2), indicating high oxidative stress (Fig. 3A), while neither of the tested exhausts E0, E10, nor E85, showed a reduction of total GSH content in the sample.

Three different genes related to oxidative stress, namely *HMOX1, SOD2,* and *GSR*, were investigated. The expression of *HMOX1* and *GSR* were significantly increased after exposure to diesel exhaust by 5.1 ± 5.4 and 1.5 ± 0.5, respectively, but not after exhausts from ethanol-gasoline blends (Fig. 3B), confirming the results of the GSH assay. On the contrary, E85 even seemed to lower the *SOD2* gene expression by 0.7 ± 0.2 compared to filtered air exposure.

Two mRNA levels of pro-inflammatory markers, *TNFα* and *IL-8,* were measured. While in *TNFα* no change in mRNA level was observed in any of the conditions (Fig. 4), *IL-8* mRNA levels were significantly increased after diesel exposure (4.3 ± 2.5). As seen with other markers, i.e. LDH-level and *SOD2*- expression, E85 reduced the RNA level of *IL-8* compared to filtered air (0.7 ± 0.5).

The additional genes tested also were not increased in gasoline-ethanol exhaust exposed cells, but exposure to diesel exhaust induced the expression of one of the three genes (*NQO1*) significantly by 2.5 ± 1.5 (Fig. 5).

The amount of DNA damage, i.e. a marker for genotoxicity, is shown in the Fig. S2. In the exhaust treated cell cultures normalized to the filtered air exposed cells, no differences could be revealed. Interestingly, also diesel exhaust did not trigger the formation of DNA damage.

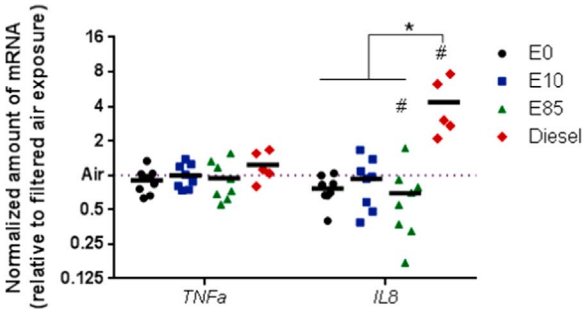

Fig. 4. Pro-inflammatory reactions in exhaust exposed lung cells. mRNA levels of two cytokines were measured, *TNFα* and *IL-8*. *TNFα* was not induced in any of the exhaust exposures; *IL-8* was increased after diesel exhaust. Data are shown as single values and mean (line);* depict differences between exposures, # between filtered air and its correlating exposure, $p < 0.05$ was considered statistically significant.

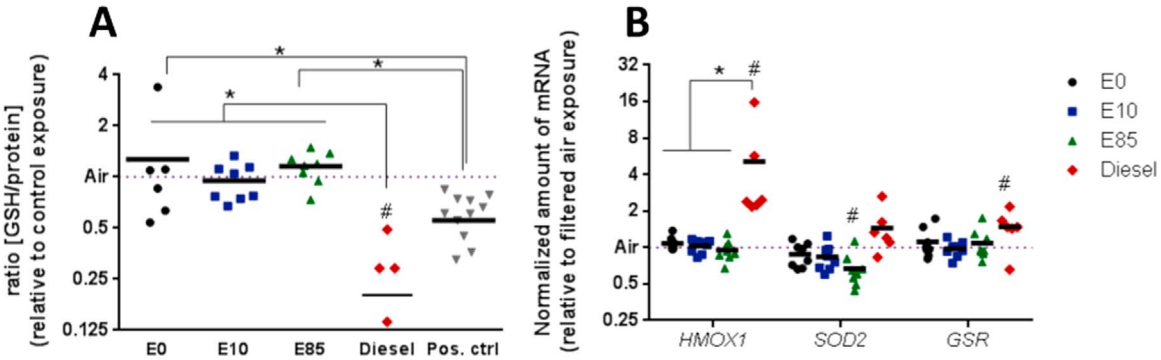

Fig. 3. Oxidative stress response in lung cells exposed to exhaust. (A) Normalized amounts of GSH, an anti-oxidative tri-peptide, are shown. Two values in diesel exhaust are cropped out of the graph (at zero). Both the positive control and diesel exhaust significantly reduced GSH, therefore induced oxidative stress. (B) Normalized amounts of mRNA of oxidative stress related genes (*GAPDH* being the standard gene), HMOX1 is significantly induced after diesel exhaust exposure. Data are shown as single values and mean (line);* depict differences between exposures, # between filtered air and its correlating exposure, $p < 0.05$ was considered statistically significant.

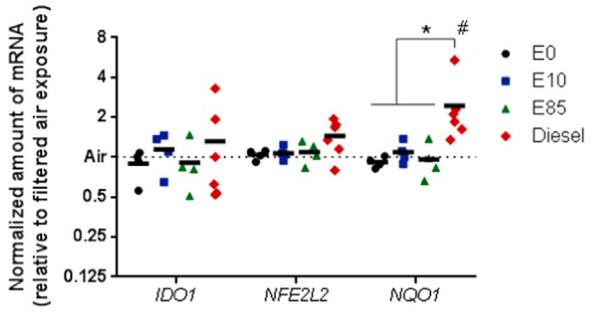

Fig. 5. Additional gene expression in lung cells. Three genes were tested, one was significantly elevated after diesel exhaust, none of them after gasoline-ethanol exhausts. Data are shown as single values and mean (line);* depict differences between exposures, # between filtered air and its correlating exposure, $p < 0.05$ was considered statistically significant.

4. Discussion

The detailed physico-chemical characterization of gasoline exhausts resulting from different fuels (gasoline E0, ethanol-gasoline mixtures E10 and E85) was determined and their possible adverse effects in a multi-cellular human lung model were evaluated using an air-liquid exposure approach. The exposure system has been established for scooter exhaust emissions (Muller et al., 2010), was then adapted to characterize diesel exhaust (Müller et al., 2011), and was recently used for filtered as well as unfiltered gasoline exhausts (Bisig et al., 2015). Different endpoints such as cytotoxicity, oxidative stress, pro-inflammation and genotoxicity were assessed after a 6 h acute exposure to 1:10 diluted whole exhaust and additional post-incubation time of 6 h. Diesel vehicle exhaust exposure was performed as a control to validate the cell system's responsiveness.

4.1. Methodological aspects and exhaust analysis

A dilution factor of 10 was chosen to be comparable to earlier studies of our and other groups, e.g. (Reed et al., 2008; Steiner et al., 2014). It is difficult to predict whether this dilution is similar to real life exposure, since tailpipe emissions don't distribute linear into the environment and depend on many parameters such as tailpipe position, velocity, other vehicles/objects close by, and weather (Chang et al., 2009; Uhrner et al., 2007). A study with a heavy-duty truck shows 10% of CO_2 approximately 0.65 m along the centerline of the plume (Kim et al., 2001). Another study on a diesel passenger car simulates and measures CO_2 and PN on the exhaust centerline (up to 1 m) on high velocities (120 km/h and 148 km/h), after one meter, still more than 10% of CO_2 and PN are measured (Uhrner et al., 2007). Conclusively, an exposure to exhaust for 6 h with a dilution factor of 10 is representing an acute rather high dose exposure, equal to a person walking/working 6 h near roadway (up to 1 m distance from the cars).

Significant PN differences in the exhausts of ethanol-gasoline blends were measured. We have used a new flex-fuel car and started the test series with E10, then E85, and ended with E0. Between these exposures, the car was not used for other experiments. It has been described that ethanol has a cleaning effect during use of the car resulting in fewer particles (Dutcher et al., 2011). We indeed have observed this phenomenon, where E10 emitted high PN on all four exposure days, cleaning the particle pool already existing in the car. Also during the first day of exposure with E85, a significant increase in PN was observed; however, from the second day of exposure with E85, almost no particles could be detected anymore also explaining the high variation for PN in the E85 condition. We therefore conclude that for future studies acclimatization should be done to get stable PN emissions. On the other side, reality is closer mimicked using the car without conditioning, since a real life flex-fuel car owner chooses fuel based on the price and does not necessarily stick to one fuel (Pacini and Silveira, 2011).

For the volatile components, i.e. CO, T.HC, and NO_x, no differences were found for the exhausts of E0, E10 and E85. This is in agreement with a more recent study, where they observed only small intra-car differences in E0, E5, and E85, though with a larger inter-car variation (Winther et al., 2012).

4.2. Linking exhaust emissions to biological effects

Particle emissions of the diesel vehicle were highest at average of 2.8×10^6 #/cm^3. Those of the flex-fuel vehicle operated with E0, E10, and E85 were on average 20-4600x lower. Because no significant effects of ethanol-gasoline blends on most biological endpoints were found, we can conclude that particle exposure levels below those of the diesel vehicle were not crucial.

Although we have seen significant PN variation in E0, E10, and E85, this did not induce adverse effects such as cytotoxicity, changed cell morphology, oxidative stress or pro-inflammation after an acute exposure of the cells for 6 h and 6 h post-incubation. Oxidative stress and pro-inflammation are key markers measured after combustion-derived nanoparticle exposures (Donaldson et al., 2005). Indeed our control exposures with diesel exhaust showed an elevated cytotoxicity, oxidative stress, and an increase in the pro-inflammatory cytokine *IL-8* mRNA expression.

We observed that exposure to E85 exhaust significantly reduced some of the endpoints such as cytotoxicity (LDH-level) and pro-inflammation (i.e. IL-8 expression) compared to filtered air (value < 1 in Figs. 3 and 4). We also observed significantly higher PN on the first day of E85-exposure, excluding these data points in Figs. 3 and 4 leads to a loss of the significance. Another possible explanation could be the fact that exhaust from E85 is very humid, i.e. 10–20% more humid than in the filtered air chamber. It was not possible to adjust the humidity in the two exposure chambers and therefore cells might have reacted differently.

The additional genes tested, namely *IDO1*, *NFE2L2*, and *NQO1* were chosen because they were linked with the Aryl hydrocarbon receptor (Kensler et al., 2007; Mezrich et al., 2010; Nebert et al., 2000), an important receptor for polyaromatic hydrocarbons (Hankinson, 1995). *IDO1* metabolizes tryptophan and was induced in dendritic cells after dioxin exposure (Stockinger et al., 2011). The nuclear transcription factor *NFE2L2* (also known as *NRF2*) plays an important role in inflammation and oxidative stress after exposure of mice to cigarette smoke (Rangasamy et al., 2004), also through the activation of *NQO1*. The important phase II enzyme, *NQO1*, has recently been shown to be induced after whole diesel exhaust (Zarcone et al., 2016), analog to our findings.

We found no increase in DNA damage, which can occur as a consequence of oxidative stress, relative to filtered air exposure. This is in agreement with the findings of ethanol- gasoline exhaust exposures, where no oxidative stress was observed, however, we could also not detect DNA damage after diesel exhaust where a strong oxidative stress was measured. It also has been proven that diesel exhaust particles can induce DNA damage in vivo (Ichinose et al., 1997). For further investigations, it would be interesting to also measure mRNA levels of repair mechanisms for DNA damage (Tsurudome et al., 1999), since 6 h are a rather short exposure, during which DNA repair mechanisms should still be intact. This might be a reason for our findings. Another reason could be an interference of the diesel particles with the assay dye (data not shown).

4.3. Comparison to other studies

Our results indicate that acute exposure with the SSC for exhausts from E0, E10, and E85 did not induce adverse cell responses for any of the conditions tested in the multi-cellular human lung model mimicking the bronchial compartment. Whether other endpoints or non-lung

related effects are impaired needs to be investigated in further studies. Other studies performed in the late 80 s using ethanol exhaust also revealed a low-toxicity compared to gasoline exhaust (Massad et al., 1985), though in the AMES test Lotfi *et al.* found ethanol exhaust as an indirect mutagen and attributed this effect to the aldehydes in the exhaust (Lotfi et al., 1990). Also in the chronic exposure study with animals, gasoline exhaust was worse than the ethanol exhaust in the assessed biological endpoints (Massad et al., 1986). It has to be noted that these studies were performed with different engine technologies and in a time with different fuel-regulations, nevertheless the authors describe the effects of the different fuels with whole exhaust generated freshly.

Though subchronic exposures of exhausts from ethanol-gasoline blends have been performed, chronic exposures with the new gasoline car technology should be considered in the future. In addition, since E85 is expected to emit acetaldehyde and formaldehyde (from unburned ethanol), both of which might increase ozone levels, a study with aged E85 exhaust should be included (Jacobson, 2007).

5. Conclusion

Impressive reductions in the emissions of toxic exhaust components by improved car technologies, implementation of particle filters as well as other after-treatment systems have been achieved in recent years. In addition, efforts are ongoing to replace petroleum-based fuels with renewable sources, which potentially further reduces toxic components of the exhaust. Because of the high complexity of engine emissions and the unpredictable exhaust composition, it is unlikely that a correlation of exhaust toxicity from exhaust composition will be possible in the near future; hence detailed toxicological studies will be required. We have shown that a multi-cellular human lung model with a direct exposure of exhaust emissions at the lung cell surface is relevant and reliable for hazard identification studies. Our results indicate that an acute exposure to diesel exhaust induced significant adverse effects which were not found after the exposure to gasoline or ethanol supplemented gasoline exhaust.

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Conflict of interest

Andreas Mayer is the owner and general manager of "TTM Andreas Mayer", Switzerland, an emission consulting company. As all the other authors however, he declares to have no conflicts of interest.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.envres.2016.09.010.

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